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Elevated expression of let-7b-3p enhances aggressiveness of larynx squamous cell carcinoma cells

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ABSTRACT

Aims: Larynx squamous cell carcinoma (LSCC) is the second most common head and neck malignancy. While let-7b-3p has been shown to have a role in cancer progression in malignancies, there is no research examining the association between LSCC and let-7b-3p. This study aimed to investigate the expression status of let-7b-3p and the potential roles of this microRNA (miRNA) in LSCC.

Methods: Using quantitative real-time polymerase chain reaction (qRT-PCR), we examined the expression status of let-7b-3p in 36 LSCC samples and the neighboring normal tissues. Then, the let-7b-3p miRNA mimic was transfected into Hep-2 cells via lipofectamine 2000 reagents. Cell viability was determined using the cell viability detection (CVDK-8) kit, and cell migration was evaluated with the scratch assay. To identify differentially expressed genes (DEGs) in larynx cancer GSE137308 and GSE130605 datasets were downloaded and reanalyzed using Gene Expression Omnibus (GEO2R) tool. Potential target genes of let-7b-3p were investigated in the miRNA target prediction and functional annotation database (miRDB). Shared genes between geo datasets and miRDB results were identified and the relationship between these genes and LSCC was investigated in the literature.

Results: We demonstrated that the expression levels of let-7b-3p was significantly upregulated in LSCC tumor tissues in comparison to the corresponding normal tissues. Mimic let-7b-3p transfection enhanced Hep-2 cell proliferation and migration. In vitro and bioinformatics analysis showed that overexpression of let-7b-3p can enhance the larynx cancer cell proliferation and migration through MYBPC1.

Conclusion: It was evaluated that let-7b-3p/MYBPC1 axis could potentially affect the LSCC process. Let-7b-3p has the potential to be a biomarker for LSCC, therefore, the let-7b-3p/ MYBPC1/LSCC relationship should be elucidated with new studies.

Keywords: Let-7b-3p, larynx squamous cell carcinoma, MYBPC1, bioinformatics

INTRODUCTION

Larynx squamous cell carcinoma (LSCC) is the second most prevalent malignant tumor in the upper aerodigestive tract, behind lung cancer. The majority of laryngeal cancers, precisely more than 90%, arise from the mucosal lining. Among these tumors, the most prevalent cytotype is distinguished squamous cell carcinoma.[1]

MicroRNAs (miRNAs) are short regulatory RNAs that consist of 18-22 nucleotides.[2,3] MiRNAs, which may exhibit either tumor suppressor or oncogenic functions, have been shown to be capable of differentiating various stages of many cancer types.[4-6] Genetic changes, such as deletion, translocation, or amplification, as well as epigenetic modifications, such as Deoxyribonucleic acid (DNA) methylation and histone modification, may either boost or reduce the levels of miRNA expression in the cell.[4,7]

For a considerable amount of time, it has been known that miRNAs consist of -5p and -3p strands, and dysregulation of these arms may be linked to a wide range of disorders.[8] The -5p strand is initially situated in the forward (5'-3') position of the precursor miRNA stem-loop structure, whereas the -3p

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strand, which will nearly complement the -5p strand, has been placed in the reverse location.[9] -5p and -3p strands may bind to distinct mRNAs and have diverse effects in the cell.[9,10] Several investigations have shown that let-7b-5p is a tumor suppressor in many cancer types.[11-13] However, the relationship between let-7b-3p and cancer has been investigated in limited studies. Therefore, the present study aimed to reveal the role of the let-7b-3p in LSCC.

METHODS

Clinical Samples

The study was conducted with the permission of the Institutional Review Ethics Committee of İstanbul University Cerrahpaşa Faculty of Medicine (Date: 23.10.2014, Decision No: 83045809/604.01/02-4221477). This study was conducted using the doctoral thesis data of the corresponding author. All procedures were carried out in accordance with the ethical rules and the principles of the Declaration of Helsinki. Informed written consents were acquired from all patients prior to their inclusion in the research. The Department of Otorhinolaryngology at Cerrahpaşa Faculty of Medicine Hospital, İstanbul University-Cerrahpaşa, collected tumor tissue and corresponding adjacent normal tissue samples from 36 individuals who underwent surgical treatment for LSCC. Following the surgery, the freshly resected normal and tumor samples were rapidly snap frozen in liquid nitrogen and stored at −80˚C until they were required. Chemotherapy, radiotherapy, or immunotherapy was administered to any of the patients who participated in the study prior to the surgical operation. The following information about the patients was presented in **Table 1**: age, gender, histological grade, and T classification. The obtained data were not subjected to clinical analysis due to the limited number of LSCC patient samples included in the study. Rather, molecular analysis data and bioinformatics analysis results were assessed concurrently.

Table 1. The patients' clinicopathological information

	LSCC Subjects	Percentage
Age		
≤60	15	41.6%
>60	21	58.3%
Gender		
Male	32	88.8%
Female	4	11.1%
T Classification		
T1 and T2	6	16.6%
T3 and T4	30	83.3%
Histological grade		
II	14	38.8%
III	22	61.1%

T: Size and extend of the tumor, LSCC: Larynx squamous cell carcinoma

Hep-2 Cell Culture

The Hep-2 cell line was obtained from the SAP Institute in Turkey, which is affiliated with the Ministry of Food, Agriculture, and Livestock. Hep-2 cells were cultured with RPMI1640 medium supplemented with 10% fetal bovine serum, 100 µg/ml streptomycin and 100 U/ml penicillin in a 5% CO2, 37°C incubator.

Let-7b-3p Mimic Transfection

Hep2 cells were seeded at 60% confluence into 96-well plates or 6-well culture plates. After 24 hours, according to the supplier's method for transient 30 nM let-7b-3p mimic or miR-NC (Thermo Fisher Scientific), were transfected via lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Cell Viability Assay

The CVDK-8 method, that colorimetrically reveals knowledge of viability, was employed to investigate the potential influence of the let-7b-3p on cell proliferation. In order to accomplish the goal, 5x103 cells were seeded in three wells of a 96-well culture plate for let-7b-3p mimic and non targeting (nt) control mimic. At 48 and 72 hours, cell proliferation was assessed by measuring absorbance at 450 nm using the CVDK-8 reagent (EcoTech Biotechnology) protocol and the MultiScan FC microplate reader (Thermo).

Scratch Assay

In order to assess the migration capabilities of Hep-2 cells transfected with let-7b-3p mimics, a scratch wound-healing method was conducted. Following the cells reached a confluency level of 95-100%, sterile 200 µl pipette tips were used to create wounds of comparable size. An inverted microscope was used to capture images of the wounds at 0 and 48 hours. The varying migration of cells was evaluated by analyzing wound closure by the measurement of gap sizes in 10 randomly chosen locations of the wound. The mean gap size was accepted as 100% at 0 hours.

RNA Isolation, Complementary DNA Synthesis and Quantitative Real Time PCR

Total RNA from tissues and Hep-2 cells were extracted using TRIzol (Invitrogen, San Diego, CA) according to the manufacturer's instructions. Spectrophotometric measurement and gel electrophoresis were used to evaluate the quantity and quality of RNA samples. Complementary DNA (cDNA) was synthesized using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) and miRNA specific primers. MiRNA expression analysis was carried out using TaqMan Universal Master Mix (Applied Biosystems) and miRNA specific probes (Applied Biosystems) were used. Quantitative real-time PCR (qRT-PCR) tests were conducted using a Roche LightCycler 480-II real-time thermal cycler (Roche, Switzerland) in accordance with established protocols. RNU43 was used for the internal control. Each experiment was conducted in duplicate. Relative expression levels were determined using the delta-delta-Ct method.

Identification of Potential Targets of Let-7b-3p

To determine Differently Expressed Genes (DEGs) associated with larynx cancer GSE130605 and GSE137308 geo datasets were used. GEO2R was utilized to analyze geo datasets. The parameters logFC>2 and p<0.01 were considered in the DEGs analysis. Since let-7b-3p expression was found to be increased in LSCC tissues potential target gene expression was expected to be decreased. Therefore, downregulated genes were considered in DEGs analysis of the geo datasets. In

silico potential target genes of let-7b-3p were investigated in the miRDB tool. Shared genes in GSE130605 and GSE137308 geo datasets and miRDB were identified.

Statistical Analysis

The data were represented as mean±standard deviation of the mean. The differences were assessed for statistical significance using Student's t-test. P values equal to or less than 0.05 were considered statistically significant. Statistical analyses were performed with GraphPad Prism 10.0 (GraphPad Software, Inc., San Diego, CA).

RESULTS

Let-7b-3p is Overexpressed in LSCC Tumor Tissue Samples

The clinicopathological characteristics of the patients are demonstrated in **Table 1**. Measurement of the expression level of let-7b-3p in LSCC samples and the corresponding adjacent normal tissue sapmles obtained from the same patients were performed using qRT-PCR. Our findings revealed that in 32 of all 36 normal-tumor tissue pairs, let-7b-3p expression was significantly upregulated in tumor samples compared to the adjacent normal tissue specimens. When all samples are considered together, let-7b-3p expression was found to be upregulated almost 1.5-fold in tumor samples compared to normal tissue specimens (**Figure 1**; p=0.004).

Let-7b-3p Enhances Hep-2 Cell Proliferation and Cancer Aggressiveness

The mimic let-7b-3p was effectively transfected into Hep-2 cells (**Figure 2A**). Ectopic expression of let-7b-3p was seen to enhance Hep-2 cell proliferation relative to the control group (**Figure 2B**). Cellular migration is a crucial determinant in the development of cancer metastases. An examination of wound healing investigated the impact of let-7b-3p overexpression on the migration of Hep-2 cells. The findings demonstrated that the wound closure rate had been significantly increased in let-7b-3p transfected cells relative to the control group (**Figure 3**).

Let-7b-3p May Influence the LSCC Cancer Process by Altering MYBPC1 Expression

The analysis of the geo datasets revealed that 549 genes were downregulated in the GSE130605 dataset and 944 genes were downregulated in the GSE137308 dataset, both of which fulfilled the criteria of logFC>, p<0.01. In the miRDB in silico tool, let-7b-3p was found to target 1770 genes. It was defined that 14 genes were shared in GSE130605, GSE137308 geo datasets and miRDB (**Figure 4** and **Table 2**).

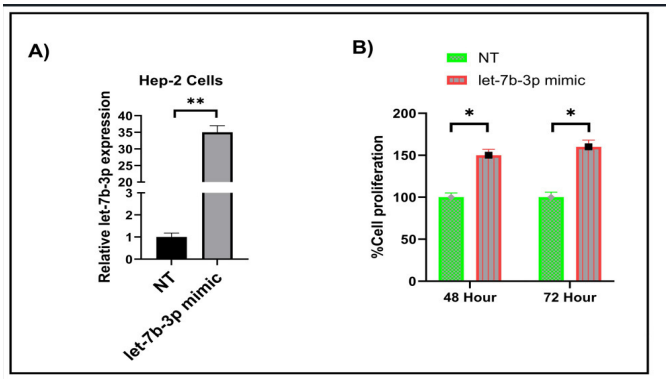

Figure 2. Effect of mimic let-7-3p transfection on Hep-2 cells. **A)** In the expression analysis performed for validation of mimic let-7b-3p transfection, a significant increase was found in mimic let-7b-3p transfected Hep-2 cells compared to the control group. **B)** In the evaluations performed at 48 and 72 hours after mimic let-7b-3p transfection, a significant increase in cell proliferation was found in the let-7b-3p mimic transfection group compared to the control group. (*: p<0,05, ** p<0,01)

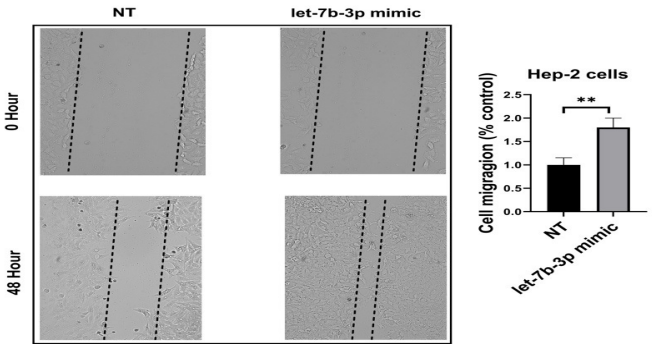

Figure 3. Overexpression of let-7b-3p was found to significantly enhance the migration of Hep-2 cells (p<0.01)

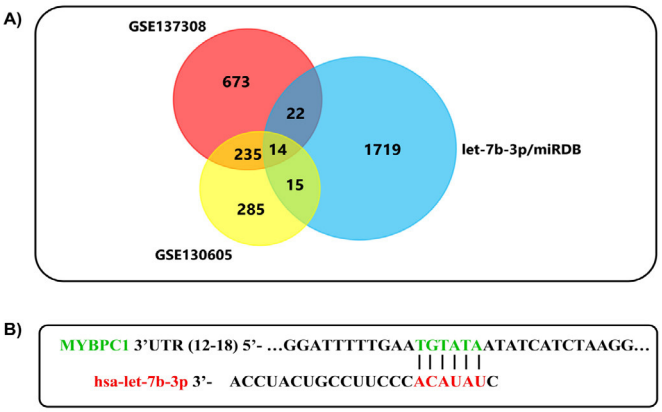

Figure 4. LSCC related genes and let-7b-3p potential target genes. **A)** Venn diagram of DEGs shared in the GSE137308 and GSE130605 geo datasets and included in the miRDB tool. **B)** Base pairing between let-7b-3p and its potential target gene MYBPC1

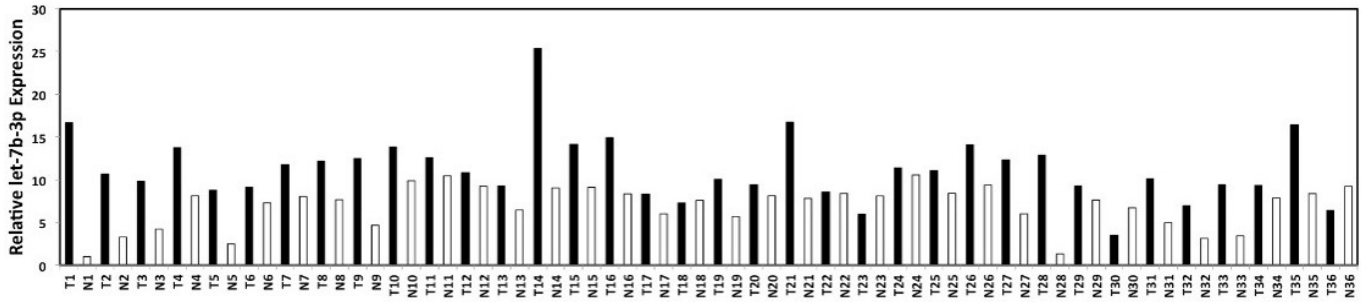

Figure 1. Relative expression of let-7b-3p in each tumor and normal laryngeal tissue specimens (T: Tumor, N: Normal)

Table 2. The expression levels of potential let-7b-3p targets in the GSE130605 and GSE137308 LogFC

	Genes	GSE130605		GSE137308	
Gene symbol	Gene name	p value	logFC	p value	**logFC**
NPY1R	Neuropeptide Y receptor Y1	7.07e-15	-3.53	1.78E-03	-4.59
BMP3	Bone morphogenetic protein 3	4.80e-18	-3.49	7.96E-05	-3.14
MYBPC1	Myosin binding protein C1	1.04e-13	-3.21	1.04E-03	-9.07
NRXN1	Neurexin 1	3.06e-21	-2.98	3.45E-05	-3.95
ESRRG	Estrogen related receptor gamma	1.88e-16	-2.92	9.74E-03	-2.49
NOVA1	NOVA alternative splicing regulator 1	2.81e-27	-2.9	4.04E-02	-2.1
NBEA	Neurobeachin	1.83e-23	-2.83	2.28E-02	-2.03
CA8	Carbonic anhydrase 8	6.40e-14	-2.68	4.26E-02	-2.3
ERBB4	Erb-b2 receptor tyrosine kinase 4	7.40e-09	-2.67	1.04E-03	-3.92
TFAP2B	Transcription factor AP-2 beta	4.09e-12	-2.56	1.67E-02	-2.84
ZMAT1	Zinc finger matrin-type 1	9.71e-39	-2.56	1.09E-01	-2.01
SLC6A4	Solute carrier family 6 member 4	1.04e-19	-2.52	1.35E-04	-4.45
PPP1R9A	Protein phosphatase 1 regulatory subunit 9A	2.47e-08	-2.12	1.33E-02	-2.78
SCN7A	Sodium voltage-gated channel alpha subunit 7	3.74e-61	-4.42	4.44E-04	-4.22

LogFC: Log fold change

DISCUSSION

To date, the dysregulations of many miRNAs have been closely related to the pathogenesis of several human cancers.[3,14-16] MiRNAs released by tumor cells into extracellular fluids, such as blood and saliva, function as signaling compounds to facilitate cell–cell contact and may serve as possible markers for cancer.[17] Thus miRNAs are becoming an increasingly popular topic of study among a variety of molecular components that have the potential to be examined for diagnostic and/or prognostic purposes.[18] A number of researchers have suggested that the let-7 family may serve as creative non-invasive biomarkers, offering promise for cancer detection. In mammals, let-7 is recognized as the regulator of differentiation, and its aberrant regulation and expression are associated with the onset and progression of cancer.[19] There are ten mature let-7 miRNAs in humans, which are generated from thirteen precursor genes. These microRNAs (let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, let-7i, miR-98, and miR-202) have critical roles in cellular functions.[20] For example, let-7b-5p has been found to inhibit breast cancer cell growth and metastasis through suppression of hexokinase 2-mediated aerobic glycolysis.[21] Bahojb et al.[22] showed that the overexpression of let-7a-3p enhances chemosensitivity to carmustine and synergistically induces autophagy while inhibiting cell survival in U87MG glioma cells. It was reported that let-7d suppresses the growth of colorectal cancer cells via the CST1/p65 pathway.[23]

In our study, we demonstrated that let-7b-3p is overexpressed in LSCC tumor tissues compared to adjacent normal tissue samples. Furthermore, it was observed that cell proliferation and migration were increased in Hep-2 cells transfected with mimic let-7b-3p in comparison to the control group. These findings suggest that let-7b-3p may function as a possible oncomiR in LSCC.

Dysregulation of let-7b-3p has been linked to several conditions. For instance, Demirel et al.[24] reported that let-7b-3p was dramatically overexpressed in the brain tissue of methamphetamine abusers. In another investigation, the expression of let-7b-3p was shown to be elevated in the patient group with mesangial proliferative glomerulonephritis.[25] Liu et al.[26] showed that the overexpression of let-7b-3p and the deregulation of nine other miRNAs were linked to myocardial ischemic reperfusion injury.

In contrast to other let-7 family members, let-7b-3p has received less attention in cancer research.[27] In one of the few research on this topic, 10 serum miRNAs (including let-7b-3p) were demonstrated as the most abundant miRNAs in the pleuro-pulmonary blastoma cases.[28] Extracellular vesicle miRNAs are critically significant in malignancies, and a recent study has shown that let-7b-3p was increased as an extracellular vesicle miRNA in patients with advanced colorectal cancer.[29] TRIM25, APP, ELAV1, RNF4, and HNRNPL' genes were identified as promising targets for Ewing sarcoma therapy in the bioinformatics analysis conducted by Weaver et al.[30] Let-7b-3p is one of the microRNAs that has the potential to have a function in the regulation of these genes. Li et al.[31] demonstrated that let-7b-3p suppresses tumor proliferation and metastasis by modulating the MAPK/ERK pathway via BRF2 in human lung adenocarcinoma. Li et al.[31] suggested that let-7b-3p might function as a tumor suppressor miRNA in human lung adenocarcinoma. However, our investigation demonstrated that let-7b-3p, which exhibited elevated expression in LSCC cancer tissue samples, enhances cell proliferation. Previous investigations have revealed that miRNAs may have opposing expressions in different malignancies. Therefore, identifying the cancer-specific expression patterns of miRNAs, arising from the intricate nature of cancer is crucial for understanding molecular pathways. To the best of our knowledge, no study has been reported regarding the relationship between LSCC and let-7b-3p.

The bioinformatic analysis of the current investigation identified 14 genes that let-7b-3p could potentially target and play a function in the LSCC process (**Table 2**). It has

been reported that each of these 14 genes are essential for the development of a variety of malignancies including breast cancer, small-cell lung cancer, and gastric cancer.[32-34] For instance, Ma et al.[35] demonstrated that ZMAT1 functions as a tumor suppressor in pancreatic ductal adenocarcinoma by triggering the SIRT3/p53 signaling pathway. Su et al.[36] found that miR-205 enhances tumor proliferation and invasion by targeting ESRRG in endometrial cancer. However, except for Myosin-binding protein C1 (MYBPC1) Pubmed database does not include any research that investigated whether or not there is an association between LSCC and other 13 genes. Thus we suggest MYBPC1 needs to be highlighted as one of the possible target genes of let-7b-3p that may be related to LSCC. MYBPC1 is a protein found in large quantities in skeletal muscle and is mostly secreted by slow-twitch muscle fibers.[37] MYBPC1 has been shown to be dysregulated in many cancers.[38-41] It has been revealed that this gene is downregulated in laryngeal carcinoma, and it has been identified as a crucial regulator of the development of oncogenesis. Furthermore, it was shown that MYBPC1 had a negative association with the stage of individuals who were diagnosed with laryngeal cancer.[38] The function of miRNAs in controlling the expression of MYBPC1 gene remains mostly unknown. The research conducted by Liu et al.[38] proposed that miR-451a may indirectly modulate the function of MYBPC1 via the ATF2-dependent signaling pathway.

The findings of our research provide clues that may be used to guide additional research into the interaction between let-7b-3p and MYBPC1 in LSCC. However, it is recommended that additional studies be conducted using the luciferase reporter assay methods etc. to validate this proposed axis.

CONCLUSION

Let-7b-3p overexpression in LSCC patient tissue samples was shown for the first time by the present study. Additionally, it has been shown that the overexpression of let-7b-3p in Hep-2 cells may lead to an increase in the proliferation and migration of cancer cells. Following that, bioinformatics approaches were used to find the possible target genes of let-7b-3p. The results of this investigation revealed that the link between let-7b-3p and MYBPC1 may be significant in LSCC. Further studies, however, are needed to elucidate the roles of let-7b-3p, in more detail in LSCC pathogenesis.

ETHICAL DECLARATIONS

Ethics Committee Approval

The study was conducted with the permission of the Institutional Review Ethics Committee of İstanbul University Cerrahpaşa Faculty of Medicine (Date: 23.10.2014, Decision No: 83045809/604.01/02-4221477).

Informed Consent

All patients signed and free and informed consent form.

Referee Evaluation Process

Externally peer-reviewed.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Financial Disclosure

The authors declared that this study has received no financial support.

Author Contributions

All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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